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The prospects for development of well defined tumor vaccines has received great impetus from the demonstration that human melanoma express common antigens recognized by cytotoxic T cells (CTL). Although significant problems related to antigen delivery and immune stimulation remain to be resolved, the availability of molecularly defined breast tumor antigens would be a major step towards development of a vaccine for breast cancer. The most successful approaches to identify antigens uniquely expressed in tumor cells employ tumor-specific cytotoxic T cells to screen a library of gene products expressed in the tumor. We have developed a novel technology for this purpose that is far more efficient and sensitive than the available alternatives. This technology is being coupled with some novel methods to generate breast tumor-specific T cells for analysis.

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FOREWORD

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Riccardo Ghetti 11/09/00

PI - Signature Date

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INTRODUCTION

The prospects for development of well defined tumor vaccines has received great impetus from the demonstration that human melanoma express common antigens recognized by cytotoxic T cells (CTL). Although significant problems related to antigen delivery and immune stimulation remain to be resolved, the availability of molecularly defined breast tumor antigens would be a major step towards development of a vaccine for breast cancer. The most successful approaches to identify antigens uniquely expressed in tumor cells employ tumor-specific cytotoxic T cells to screen a library of gene products expressed in the tumor. We have developed a novel technology for this purpose that is far more efficient and sensitive than the available alternatives. This technology is being coupled with some novel methods to generate breast tumor-specific T cells for analysis.

BODY OF PROGRESS REPORT

In abbreviated form, the three major tasks of the approved statement of work are: 1) construction of a representative human breast tumor cDNA library in a modified vaccina virus vector; 2) development of a strategy to generate human tumor-specific T cells; and 3) isolation of cDNA encoding the target antigens of these breast tumor-specific T cells by using a novel and proprietary selection method described in the application.

As anticipated from previous work in an animal tumor model, no difficulties were encountered in accomplishing Task 1, the construction of a human breast tumor cDNA library in vaccinia vector. To insure that this library is representative of all available T cell epitopes, cDNA was synthesized employing both oligo-dT and random primers. Our concern was that oligo-dT primed cDNA might not extend to the end of the 5' coding region of long mRNA species. Since cytotoxic T cell epitopes are expected to be distributed throughout the length of this coding sequence, this could result in epitope "holes" in the library. Use of random primers is expected to capture coding sequences throughout the length of the mRNA. In order to insure that these cDNA fragments will be properly expressed and processed, three additional plasmid transfer vectors have been constructed with ATG translational start and termination codons in all three reading frames. Task 1 was completed as planned in the first 6 months of effort.

Two strategies were contemplated to achieve Task 2, selection of human tumor-specific T cells. One strategy focuses on immunization of HLA and human CD8 double transgenic mice to generate murine cytotoxic T cells (CTL) specific for human breast tumors. This strategy had the shortcoming that it does not establish whether human T cells would also be reactive to the human target antigens recognized by the murine immune system. This is a fundamental requirement if these antigens are to be useful for immunotherapy in humans. We have addressed this issue by developing an *in vitro* protocol to determine whether any given antigen is immunogenic for human T cells (see Task 2a below). In addition, we developed an alternative strategy employing human rather than murine T cells from the start. As described in our original application, we have available paired samples of human tumor and normal breast cancer tissues and cell lines. We reasoned that if human T cells could be tolerized to antigens of the normal tissue and cells, then subsequent stimulation with the tumor material should give

rise to a tumor-specific response. A variety of in vitro tolerizaton protocols were evaluated for this purpose. One, particularly effective strategy, is described below (Task 2b).

Task 3 is scheduled for the next phase of this project. We have, however, addresses a technical issue that might have impeded progress in accomplishing this task. Some of the T cell lines we are generating have either weak or no cytolytic activity. In order to screen the breast tumor cDNA library with these T cells, an alternative readout for T cell activation is required. Induction of IFN gamma secretion is one such widely employed measure. However, this cytokine is not optimal for screening a library constructed in our vaccinia virus vector because vaccinia virus expresses high levels of a viral IFN gamma receptor that competes for cytokine in the activation assay. We have determined that this results in irregular and often unreliable signals. TNF alpha secretion proved unsatisfactory for the same reason as IFN gamma, vaccinia virus also expresses a TNF alpha receptor. Further investigation has established that secretion of GM-CSF can be detected with equal efficiency in either the presence or absence of infectious vaccinia virus. The virus apparently does not have a receptor for this cytokine. In the next phase of this research, we will continue to generate human breast tumor-specific T cells and initiate Task 3, identification of the target antigens.

Task 2a. Demonstration of the immunogenicity of a selected human breast-tumor specific antigen *in vitro.*

We have identified an antigen, termed C35, that is overexpressed in 70% of human breast carcinoma. In order to evaluate C35 as a candidate for immunotherapy, it was necessary to establish that human T cells are not tolerant to this self-antigen. The only way to definitively exclude tolerance is to demonstrate responsiveness. The most efficient and reliable way to do this, short of a clinical trial, is to employ human dendritic cells (DC) as APC for stimulation of a primary response by autologous T cells *in vitro.* Since dendritic cells are not easily transfected with foreign DNA, we constructed C35 recombinants in vaccinia (1), retroviral (2), and adenoviral (3) vectors for infection of human DC. Others have shown that by alternating cycles of T cell stimulation with DC infected with the same recombinant gene in different vectors it is possible to significantly reduce the otherwise strong anti-vector response and promote outgrowth of CTL specific for the recombinant gene (4). DC from an HLA-A2, A11; B8, B35 normal donor were infected with C35

recombinant retrovirus and employed to stimulate autologous T cells *in vitro*. After 12 days, T cells were restimulated in the presence of IL-2, IL-12, and IL-18 with DC from the same donor infected with a vaccinia virus recombinant of C35. A third cycle of stimulation was then carried out with DC infected with an adenoviral C35 recombinant. The donor of DC and T cells in this experiment shares expression of HLA-A2 and B35 with a C35 positive tumor cell line (21NT) and a line of immortalized C35 negative normal breast epithelium from the same patient (H16N2). Table 1 and Figure 1 demonstrate specific lysis of tumor but not normal target cells by the T cells stimulated *in vitro* with DC infected by C35 recombinant vectors. Note that induction of *de novo* allogeneic responses due to known HLA mismatches between the target cells and the T cell donor are not a concern during this short 4 hour CML. It remains to be determined whether these T cells recognize C35 peptide epitopes in association with either HLA-A2 or HLA-B35 or both.

Table 1: Induction of a primary *in vitro* C35-specific human T cell response.

	Effector:Target Ratio			
	20:1	10:1	5:1	2.5:1
Target		(% specific lysis)		
Tumor (C35+) (HLA-A2, B35)	**49**	**36**	**30**	**16**
Normal (C35-) (HLA-A2, B35)	15	13	11	6
K562	3	3	0	0

This protocol to demonstrate immune responsiveness is especially useful because different allogeneic donors of DC and T cells can be selected to determine whether immunogenic peptides of C35 can be processed and presented in association with different polymorphic HLA molecules.

Task 2b. Induction of human tumor-specific T cells following depletion of T cells reactive to normal tissue antigens.

This strategy exploits the observation of Zhou *et al.* (5) that incubation of activated but not naive T cells with bisindolylmaleimide VIII (BisVIII, Alexis Biochemicals, San Diego) promotes activation induced cell

death. The mechanism of action is believed to be through interference with inhibitors of apoptosis. Bis VIII and other bisindolylmaleimides are potent inhibitors of protein kinase C (PKC), a family of molecules known to mediate anti-apoptotic effects in many cell types. However, not all PKC inhibitors block apoptosis, suggesting that there may be additional targets of BisVIII. For this experiment, naïve T cells were first activated in the presence of dendritic cells (DC) pulsed with a normal cell line that had been treated with anti-Fas antibody to induce apoptosis and promote antigen uptake. After 24 hours activation 10 μM Bis VIII was added for an additional 24 hours as described in the detailed protocol of Appendix A. This T cell population, depleted of reactivity to normal cellular antigens, was subsequently stimulated with DC pulsed with tumor cells that had also been treated with anti-Fas antibody to induce apoptosis and promote tumor antigen uptake by DC. Importantly, the tumor cells derive from the same individual from whom normal cells were obtained. As shown in Figure 2, T cells recovered following several cycles of stimulation are specific for two different tumor lines but not for normal epithelial cells derived from that same individual. To our knowledge, this is the first report of successful isolation of tumor-specific T cells following *in vitro* tolerization to normal cellular antigens.

Task 3. Vaccinia infection does not interfere with GM-CSF assay of T cell activation.

For purposes of this experiment a line of melanoma-specific T cells was employed to confirm that the strategy to be implemented in Task 3 is feasible with human T cells and tumor cells. It is also demonstrated that infection with vaccinia virus does not interfere with use of GM-CSF secretion as an assay for specific T cell activation. The protocol for isolation of a viral recombinant that encodes the target antigen of these melanoma-specific T cells is outlined in Appendix 2. Table 2 demonstrates specificity of TIL1700 (tumor infiltrating lymphocytes sample 1700) for the recombinant gene expressed in 20 individual viral plaques isolated from each of two 5 pfu pools (B2 and F8) that scored positive in the previous round of assays. In both cases, 6 plaques were strongly positive when used to infect autologous EBV-B1700 cells for stimulation of TIL1700. The results for individual recombinants following plaque purification are shown in Figure 3.

Table 2: Isolation of recombinant tumor antigen employing GM-CSF assay.

Sample	OD Plate 1	OD Plate 2	GM-CSF
T cells only	0.035	0.077	
Tumor only	0.005	0.017	
Tumor + TIL1700	1.525	1.482	1000pg/ml
Tumor + wtVV	0	0.002	
Tumor + wtVV + TIL1700	1.627	1.552	1000pg/ml
EBVB only	0	0.004	
EBV+ TIL1700	0.153	0.147	60pg/ml
EBV + wtVV only	0	0	
EBV+ wtVV + TIL1700	0.129	0.123	60pg/ml
(wt = empty vector)			
All of the following samples are + TIL1700			
EBV + Pool B2 (5pfu)	0.841	0.758	500pg/ml
EBV + Clone B2.5	1.566	1.526	1000pg/ml
EBV + Clone B2.6	1.582	1.481	1000pg/ml
EBV + Clone B2.7	1.515	1.429	1000pg/ml
EBV + Clone B2.9	1.466	1.421	1000pg/ml
EBV + Clone B2.11	1.473	1.478	1000pg/ml
EBV + Clone B2.12	1.467	1.491	1000pg/ml
EBV + Clone B2. 4	0.138	0.150	60pg/ml
(B2.4 is representative of 14 negative subclones)			
EBV + Pool F8 (5pfu)	0.599	0.539	350pg/ml
EBV + Clone F8.1	0.849	0.804	500pg/ml
EBV + Clone F8.3	1.361	1.259	900pg/ml
EBV + Clone F8.7	1.500	1.420	1000pg/ml
EBV + Clone F8.15	1.422	1.399	900pg/ml
EBV + Clone F8.19	1.364	1.365	900pg/ml
EBV + Clone F8.20	1.310	1.263	900pg/ml
EBV + Clone F8.9	0.160	0.143	60pg/ml
(F8.9 is representative of another 14 negative subclones)			

Figure 1. Induction of Primary *in vitro* C35-specific Human Cytotoxic T Cell Response

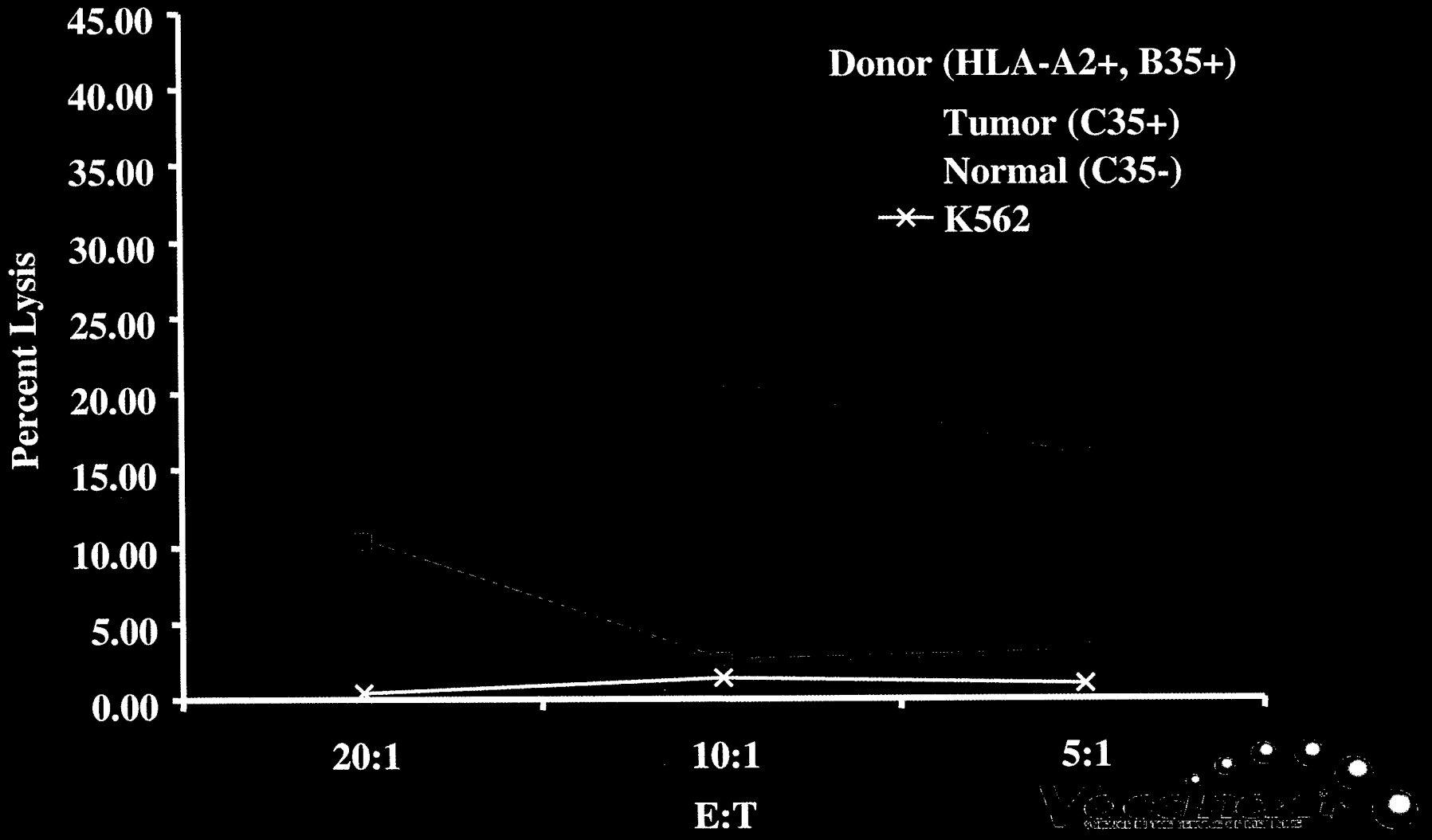


Fig. 2 Induction of tumor-specific T cells following Bis VIII mediated depletion of normal cell reactivity

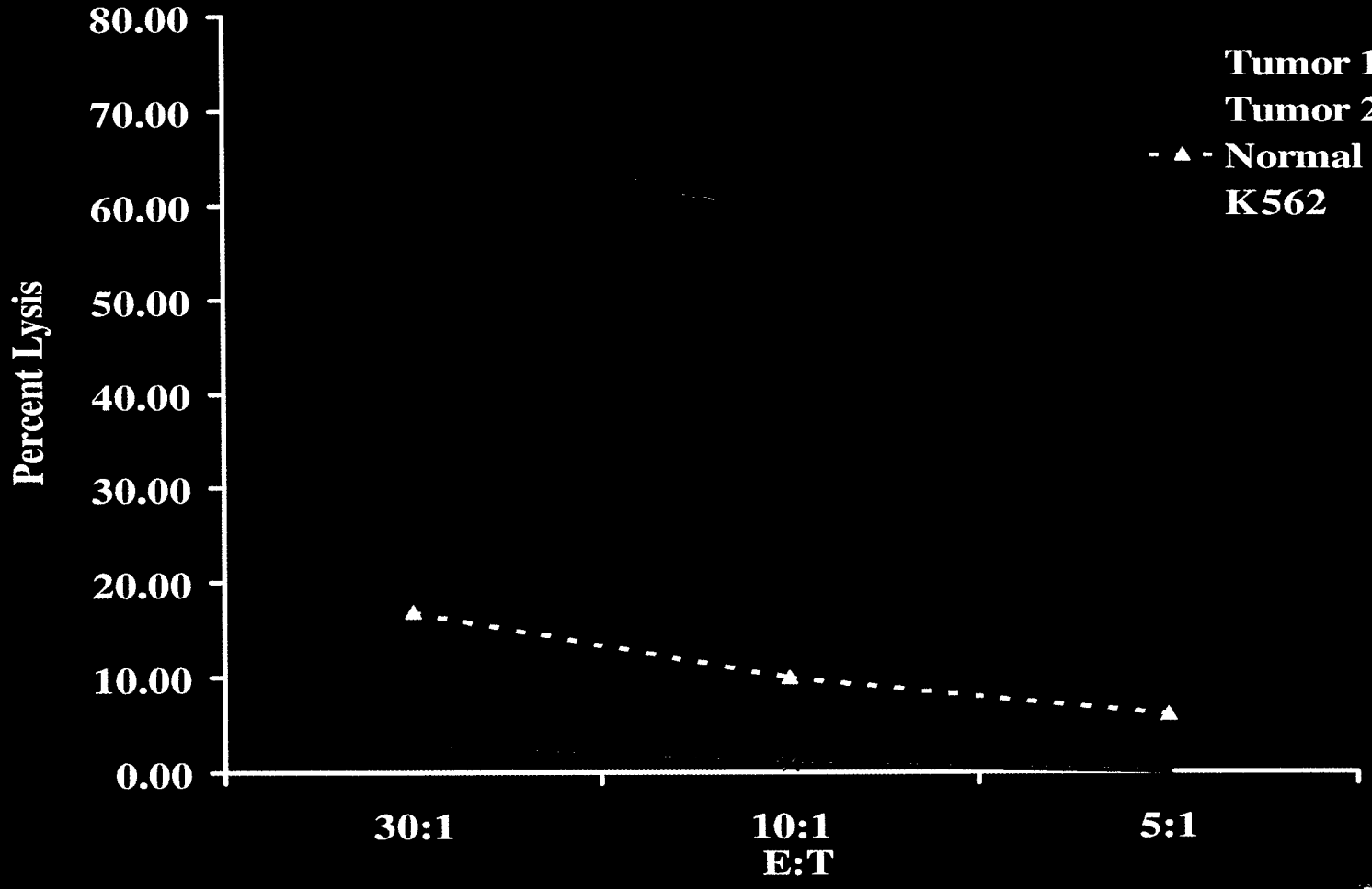
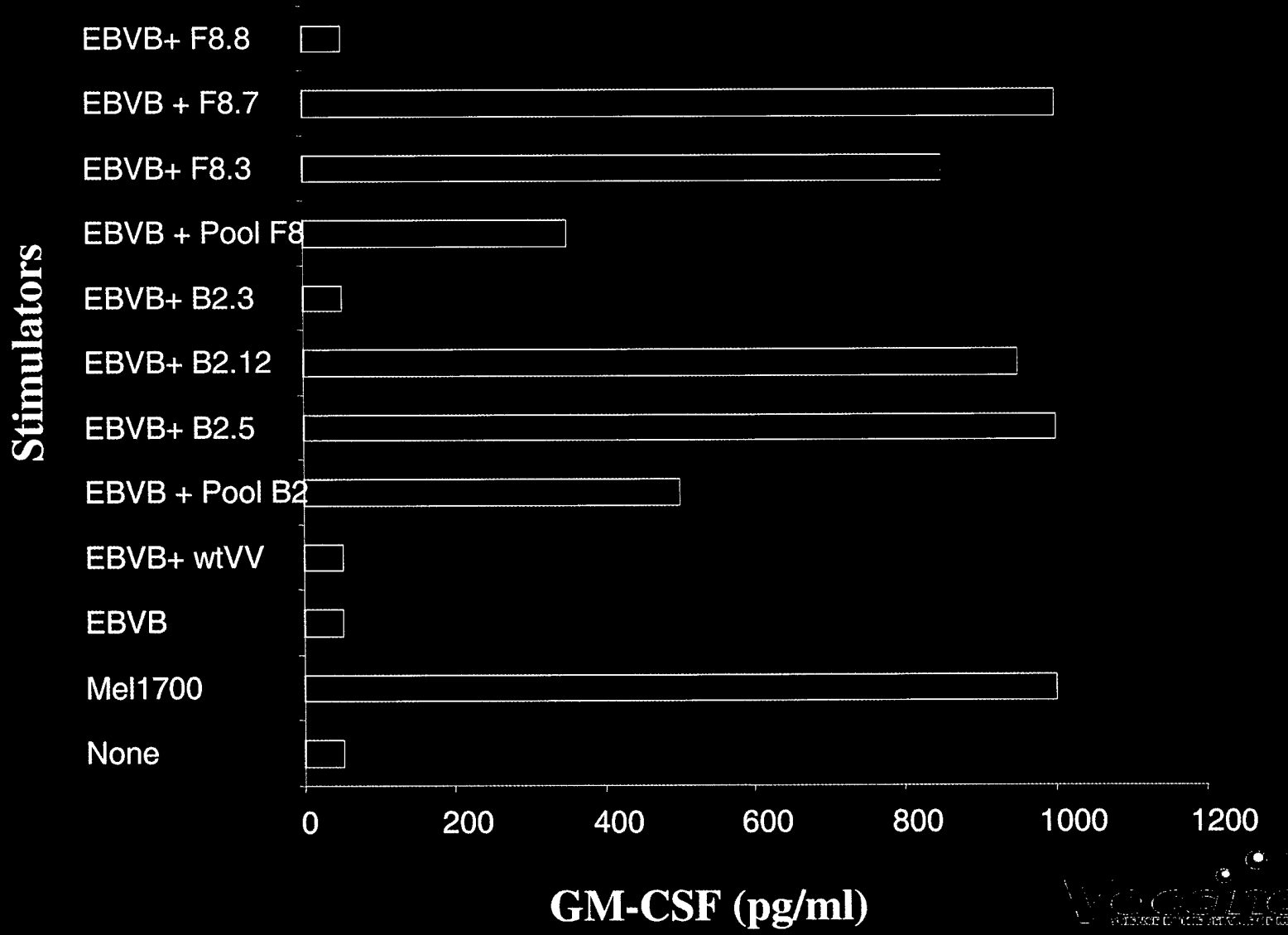


Fig. 3 Recombinant Vaccinia Clones Stimulate MEL1700 Specific TIL



KEY RESEARCH ACCOMPLISHMENTS

- Representative libraries of tumor cDNA have been constructed in a novel vaccinia based vector.

- A method has been established to demonstrate the immunogenicity of a new antigen expressed in 70% of human breast carcinoma. This same method will be applicable to new antigens as they continue to be identified.

- A new method has been developed to induce tumor-specific T cell responses *in vitro* following Bis VIII mediated tolerization of T cells specific for normal human tissue antigens. These T cells will be the raw material employed for identification of targeted breast cancer antigens using our proprietary technology.

REPORTABLE OUTCOMES

Abstracts:

Cancer Vaccines 2000, Meeting Abstract No. P-115
"A Novel Tumor Antigen Expressed in Human Breast and Bladder Carcinoma"

Cancer Vaccines 2000, Meeting Abstract No. P-116
"Application of Novel Antigen Discovery Technology to Identification of a Shared Tumor Rejection Antigen."

Cancer Colloquium, University of Rochester
"A Widely Expressed Tumor Surface Antigen of Human Breast Carcinoma."

American Association for Cancer Research #3123
"Identification of Differentially Expressed Genes"

Presentations:

Cancer Vaccines 2000

State University of New York, Upstate Medical Center, Department of Microbiology and immunology

Roswell Park Cancer Institute, Department of Immunology

Purdue Pharmaceuticals

MedImmune

Oxxon Pharmaceuticals

Genencor International

Human Genome Science

CONCLUSIONS

The feasibility of major goals of this project has been demonstrated. The supporting technology has been further enhanced and developed. A particularly exciting result was obtained employing a test population of tumor-specific cells for which other investigators had failed to identify a target antigen in spite of having screened over 600,000 cDNA clones. Employing our proprietary technology, it was possible to identify this antigen with only two weeks of effort. It appears that the key to this success was the high levels of expression of recombinant genes in the vaccinia based vector and the ease with which this infectious vector can be employed to introduce genes into autologous antigen presenting cells.

This identification of multiple new antigens associated with human breast cancer will create new opportunities for immunotherapy and early diagnosis of this excessively prevalent disease.

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APPENDIX A

Generation of tumor-specific T cells from an allogeneic donor by prior depletion of T cells reactive to normal cellular antigens using the PKC inhibitor Bis VIII.

1. Collect PBL from donor and fractionate on ficoll

2. Enrich for T cells and dendritic cells:

2.1. Form rosettes with neuraminidase treated-SRBCs. The ER+ (rosetted cells) are enriched for T cells and are cryopreserved for later use. The non-rosetted (ER-) cells are depleted of T cells and can be used to generate dendritic cells (DC).

2.2. T cells and DC may also be fractionated by magnetic activated cell sorting (MACS). PBL are stained with a cocktail of antibodies (Miltenyi Biotec Pan T cell selection kit) that allow for the depletion of non -T cells. Untouched (non-labeled) T cells pass through the column and are cryopreserved. The column retained (T cell depleted fraction) cells are then removed from the column and used to generate DCs just like the ER- fraction above.

3. Generation of DCs

3.1. ER- (or T cell depleted) cells are set up in 6 well plates at 2-3x 10^6 cells/well in 3 ml RPMI media plus 1% heat-inactivated autologous serum, 1000 U/ml GM-CSF and 1000 U/ml IL-4.

3.2. Incubate cells at 37°C, 5% CO_2 for 7 days. Replenish with complete cytokines and media every other day.

3.3. On day 7 harvest immature DCs by collecting the floating cells.

4. Depletion of T cells reactive to normal cellular antigens

4.1. To induce apoptosis, tumor cells are incubated overnight with anti-Fas antibody. Apoptosis can be confirmed using the Annexin V kit from Pharmingen and a flow cytometer. Anti-Fas treated cells are washed 3 times before pulsing DCs.

4.2. Incubate 5 x 10^4 DCs with apoptotic normal cells (1:2) in RPMI + 1% autologous serum, 1000 u/ml GM-CSF and 1000 U/ml IL-4 for 4 hours at 37°C, 5% CO_2.

4.3. Add 12.5 % monocyte conditioned media (MCM) to the DC/tumor along with 4x10^6 T cells in two wells for the Bis treated cells and 2x10^6 cells in one well for a control (no Bis). Add IL-2 (20U/ml).

4.4. Incubate 24 hours at 37°C, 5% CO_2 then add 10uM BisVIII (bisindolylmaleimide VIII, Alexis Biochemicals, San Diego) in

PBS/10%DMSO to the test wells and an equivalent volume of PBS/10% DMSO to the control wells for 24 hours at 37°C.

4.5. Check T cells for evidence of BisVIII induced apoptosis by flow cytometry and Annexin V.

5. Rescue of tumor-specific T cells by Pulsing DCs with apoptotic tumor cells

5.1. To induce apoptosis, tumor cells are incubated overnight with anti-Fas antibody. Apoptosis can be confirmed using the Annexin V kit from Pharmingen and a flow cytometer. Anti-Fas treated cells are washed 3 times before pulsing DCs.

5.2. Incubate 5 x 10^4 DCs apoptotic tumor cells (1:2) in RPMI + 1% autologous serum, 1000 u/ml GM-CSF and 1000 U/ml IL-4 for 4 hours at 37°C, 5% CO_2.

5.3. Add 12.5 % monocyte conditioned media (MCM) and CD40L (1ug/ml) to the DC/tumor along with 10^6 T cells. Add IL-2 (20U/ml) and IL-7 (10ng/ml).

5.4. Incubate 12 days at 37°C, 5% CO_2

6. CD8 selection

6.1. After a few stimulations with DCs/tumors, CD8+ cells are selected using a CD8 enrichment column from Miltenyi Biotec.

6.2. CD8+ T cells may continue to be stimulated with DCs pulsed with apoptotic tumor cells as above. May also stimulate T cells with irradiated tumor cells and irradiated pooled allogeneic PBL if DCs are unavailable (stimulate every 7-10 days instead of 12 in this case). This will work well with tumors that express B7.1.

7. CTL assays and cloning:

Repeat stimulations every 12 days with DCs until enough T cells are generated to perform ^{51}Cr release assays and/or clone T cells by limiting dilution assays (Plate T cells at 0.5 cells/well in 96-well U-bottom plates with RPMI+10% human serum, IL-2 (100 u/ml), IL-7 (10ng/ml), irradiated tumor (10^4/ml) and irradiated pooled allogeneic MNCs (10^5/ml). Or test for IFNg secretion.

Appendix B

Screening of Vaccinia Virus cDNA Library with Tumor Specific T Cells

1. Seed library at 50pfu/pool. Dilute primary library to 50 pfu/100µl using DMEM 2.5% FBS. Infect confluent monolayers of BSC1 cells in 96 well flat bottom plate with 100µl virus. Final volume in each well is 200 µl. Each plate will contain ~5,000 pfu. Incubate plates at 37°C for 2 days.
2. Release virus from cells by 3 cycles of freeze/thaw. The easiest way to do this is to leave the samples in the 96 well plate(s). Freeze plate by placing it onto dry ice. Leave on dry ice for >30 minutes to ensure that the samples are completely frozen. Thaw plate at 37°C by placing into the CO2 incubator. It takes ~30 minutes for the plate to thaw. After the 1st freeze/thaw, pipet cells/virus up and down >5X. This is to make sure that all of the cells are detached from the plate. Then perform the 2 remaining freeze/thaw cycles. Virus should be stored at –80 after use.
3. Seed 25,000 EBVB (or any other APC) into each well of a 96 well round bottom plate in a 100µl volume. EBVB are seeded in RPMI 10% FBS. Use Falcon plates with a low evaporation lid.
4. Infect EBVB with 30µl of each viral pool (step2). This corresponds to an MOI (multiplicity of infection) of 2 – 5 pfu/cell. Incubate at 37 °C for 2-3 hours.
5. Add 30,000 to 60,000 T cells in 100 µl to each well. During step 4 the T cells are counted, centrifuged, and resuspended in AIMV media containing 10 % Human Serum. Add T cells to the infected EBVB and mix cells by pipeting. Final volume in each well is 230µl. Incubate 37°C O/N.
6. Harvest supernatants at anywhere from 12-18 hours after T cells are added. To harvest supernatant, spin plate(s) 700 rpm for 5 minutes. Harvest 165 µl supernatant from each well. Spin supernatants at 700 rpm for 5 minutes to minimize the chance of carrying over cells.
7. Assay for GM-CSF by ELISA. We use "OptEIA set" from Pharmingen. For each sample, 50µl supernatant is spotted in duplicate. This leaves 50µl in case the assay needs to be repeated. The reserve supernatant is stored at –20°C. Any positive pool(s) are subcloned to 5 pfu/pool and the assay is repeated.
8. Titer any positive pool (virus from step 2). Dilute positive pool to 5 pfu/100µl. Infect BSC1 as described in step 1. Infect for 3 days (goal is to induce ~50% cytopathic effects).
9. Harvest pools as described in step 2. Repeat infection/ELISA as described in Steps 3-7. This time use 50µl virus/sample. Pick plaques from any positive subpool.
10. Seed dilutions of any positive subpool (10^{-2} to 10^{-5}) onto BSC1 cells in a 6 well plate. Use 2 wells per dilution. Incubate at 37°C for 2 days.

11. Pick 20 plaques for each subpool. Freeze/thaw 3x. Infect BSC1 cells in 96 well plate with 50% of each plaque. 1 plaque/well. Final volume is 200µl . Incubate at 37°C for 2 days.
12. Harvest cells by pipeting. Transfer to 1.5 ml tube. Freeze thaw 3x. Repeat infection/ELISA as described in steps 3-7. Except, use 50µl virus/sample.
13. Positive clones are amplified on BSC1 cells in 12 well plate for 2 days. DNA is harvested using QIAamp DNA Blood Kit (Qiagen). Inserts are analyzed by PCR using vaccinia thymidine kinase specific primers. This PCR product is used for sequencing, cloning, as probe for northern analysis, etc.
14. Positive clones are tested in a chromium release assay for the ability to sensitize targets to lysis by antigen-specific T cells.